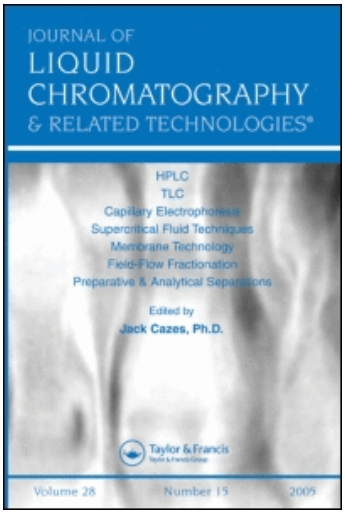

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HPLC OF (±)-FENFLURAMINE AND PHENTERMINE IN PLASMA AFTER DERIVATIZATION WITH DANSYL CHLORIDE

Amal Kaddoumi[a]; Mihoko N. Nakashima[a]; Mitsuhiro Wada[b]; Naotaka Kuroda[b]; Yuji Nakahara[c]; Kenichiro Nakashima[a]
[a] Graduate School of Pharmaceutical Sciences, Nagasaki, Japan [b] School of Pharmaceutical Sciences, Nagasaki University, Nagasaki, Japan [c] National Institute of Health Sciences, Tokyo, Japan

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HPLC OF (±)-FENFLURAMINE AND PHENTERMINE IN PLASMA AFTER DERIVATIZATION WITH DANSYL CHLORIDE

Amal Kaddoumi,[1] Mihoko N. Nakashima,[1] Mitsuhiro Wada,[2] Naotaka Kuroda,[2] Yuji Nakahara,[3] and Kenichiro Nakashima[1,*]

[1]Graduate School of Pharmaceutical Sciences and [2]School of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852-8521, Japan
[3]National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-Ku, Tokyo 158-8501, Japan

ABSTRACT

A high-performance liquid chromatographic method is described for the simultaneous determination of the dansyl derivatives of (±)-fenfluramine (Fen) and phentermine (Phen) in addition to four other sympathomimetic amines, namely, norephedrine (NE), ephedrine (E), 2-phenylethylamine (2-PEA), and 4-bromo-2,5-dimethoxyphenethylamine (2-CB), using fluoxetine (FLX) as an internal standard. The separation was performed on a reversed-phase C_{18} column, using a gradient system and fluorescence detection. The dansylation conditions were examined and the optimum

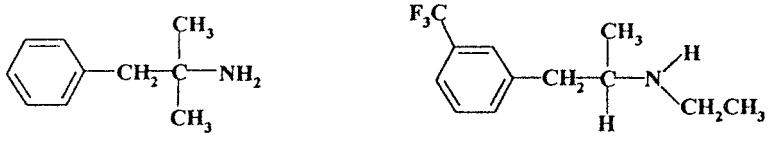
*Corresponding author.

derivatization was obtained at pH 9.0 with 4.7 m*M* dansyl chloride concentration. A preliminary study for the simultaneous determination of the dansyl derivatives in spiked human plasma was performed. The derivatives were well separated within 45 min and showed good linearities from 0.005 to 2μM for E, Phen, and Fen, from 0.002 to 0.8 μM for 2-CB, and from 0.025 to 2 μM for NE and 2-PEA with detection limits ranging from 16 to 255 fmol on the column. Extraction recovery of the compounds was greater than 90%. The applicability of the method was studied in rat plasma following an intraperitoneal administration of Fen and Phen.

INTRODUCTION

(±)-Fenfluramine (Fen) and phentermine (Phen) have been widely prescribed in combination for short-term management of obesity (1). Both drugs are phenylethylamine derivatives (Fig. 1): Fen exerts its appetite-suppressing effect through stimulating the release of serotonin and Phen through the release of dopamine and norepinephrine (1). However, the association of serious side effects, including valvular heart disease and primary pulmonary hypertension (2), with such drugs led to increased interest in Fen and Phen and necessitated the development of sensitive and precise methods for their determination in biological fluids.

Several methods have been developed for determining Phen and Fen in biological fluids, using either gas chromatography (GC) (3–6) or high-performance liquid chromatography (HPLC) (7–12). In these methods, the minimum sample volume used for their quantitation in plasma or urine was 1 mL with minimum quantitation limits of 10 ng/mL (67 n*M*) and 2.5 ng/mL (11 n*M*) for Phen (12) and Fen (3), respectively. Clausing et al. (13) described an HPLC method using dansyl chloride (Dns-Cl) as a label for determining *d*-Fen and fluoxetine (FLX) in plasma. Although the method was sensitive with a quantitation limit of 10 n*M*



Phentermine Fenfluramine

Figure 1. Structures of phentermine and fenfluramine.

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using a small volume of plasma sample (100 μL), the sample preparation needed a cleanup step to remove the excess Dns-Cl after 4 h of incubation.

In this paper, a simple, sensitive, and rapid HPLC method for the simultaneous determination of Phen and Fen, in addition to four other sympathomimetic amines, using Dns-Cl as a derivatizing reagent, is described. The method was also applied for the determination of Phen and Fen in human and rat plasma.

EXPERIMENTAL

Chemicals

(±)-Fenfluramine hydrochloride and phentermine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA), fluoxetine hydrochloride was obtained from Tocris Cookson Ltd (Bristol, UK). Ephedrine was from Dainippon Pharmacy (Osaka, Japan), 2-phenylethylamine was from Nacalai Tesque (Kyoto, Japan), norephedrine was from Aldrich (Milwaukee WI, USA) and 4-bromo-2,5-dimethoxyphenethylamine (2-CB) was synthesized by one of the authors (Y.N.) with the allowance of National Institute of Health Sciences, Japan, according to DeRuiter et al. (14).

Dansyl chloride, ethylacetate, acetonitrile, and methanol of HPLC grade were obtained from Wako Pure Chemical Ind. (Osaka, Japan). Water was deionized and passed through a water purification system (Pure Line WL21P; Yamato Kagaku, Tokyo, Japan). Other reagents were of analytical grade.

HPLC Apparatus and Chromatographic Conditions

Analysis of the compounds in this study was performed using a HPLC system (Shimadzu, Kyoto, Japan) consisting of two pumps (LC-6A) with a system controller (SCL-6A), fluorescence detector (RF-550), and a recorder (R-112).

The injector used was a Rheodyne 7125 (Rheodyne, Cotati, CA, USA) with a 20-μL sample loop. For separation, a Diasopak SP-120-5-ODS column (250 × 4.6 mm, inner diameter 5 μm; Daiso, Osaka, Japan) was used.

The mobile phases were mixtures of A (methanol/0.1 *M* acetic acid, 6:4, v/v) and B (acetonitrile). The flow rate was 1.0 mL/min. The detector was set at an excitation wavelength of 325 nm and an emission wavelength of 530 nm.

The time program for the gradient elution was set as follows: from 0 to 20 min, mobile phase B was 20% and increased to 55% from 20 to 22 min. Then mobile phase B was maintained at 55% until 39 min, where mobile phase B was programmed to return to the initial condition (20%).

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Plasma Samples

Human blood samples were drawn from healthy volunteers in our laboratory. Male wistar rats (290–370 g) were used in the experiment. Blood samples were centrifuged at 1000*g* for 10 min and the plasma samples obtained were kept frozen at −20°C before use.

Plasma Sample Extraction

The extraction was done as described by Clausing et al. (13) with a slight modification. To 100 μL of plasma, 10 μL of an aqueous solution of 2 μM FLX as an internal standard were added and mixed, followed by the addition of 200 μL of 0.1 *M* borate buffer (pH 10.6) and 750 μL of ethyl acetate. Samples were mixed on a vortex for 1 min and centrifuged for 10 min at 1700*g* and 4°C. To 600 μL of the organic layer in a vial, 10 μL of acetic acid were added and then the sample was evaporated to dryness by a centrifugal evaporator (CE1; Hitachi, Japan) for 15 min at 45°C. Samples were then derivatized with Dns-Cl as described below.

Derivatization with Dns-Cl

The residues of the evaporated methanolic standards or extracted plasma samples were derivatized as follow: 75 μL of 4.7 m*M* Dns-Cl in acetonitrile and 25 μL of 0.02 *M* carbonate buffer (pH 9.0) were added to the residue, mixed on a vortex, and then incubated at 45°C for 30 min. The reaction was stopped by adding 3 μL of 20% methylamine in acetonitrile, and left standing for 10 min followed by the addition of 4 μL of 3 *M* HCl. Twenty microliters of the resultant solution was injected onto the column.

RESULTS AND DISCUSSION

Derivatization Conditions

Initially, the following dansylation conditions were examined: Dns-Cl concentration, carbonate buffer concentration, buffer pH, and reaction temperature, as well as reaction time.

Acetonitrile was used to dissolve Dns-Cl. Derivatization was studied at different concentrations of Dns-Cl. Figure 2A shows the fluorescence intensities represented by the peak heights of Dns derivatives of the compounds studied at different Dns-Cl concentrations. In the range from 3.3 to 4.7 m*M*, no

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significant change was observed; thus, 4.7 m*M* was selected for the following experiments.

Carbonate buffer concentration was examined in the range of 0.01–0.1 *M* (Fig. 2B). Maximum and constant peak heights were obtained in the range from 0.015 to 0.04 *M* for the compounds studied. Therefore, a concentration of 0.02 *M* was used. The pH of the reaction solution is an important factor for dansylation. The pH values studied were 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0. For Phen and Fen, dansylation increased with pH; however, at the same time the formation of dansyl OH also increased, which interfered with some of the target compounds including norephedrine (NE), ephedrine (E), and 2-phenylethylamine (2-PEA).

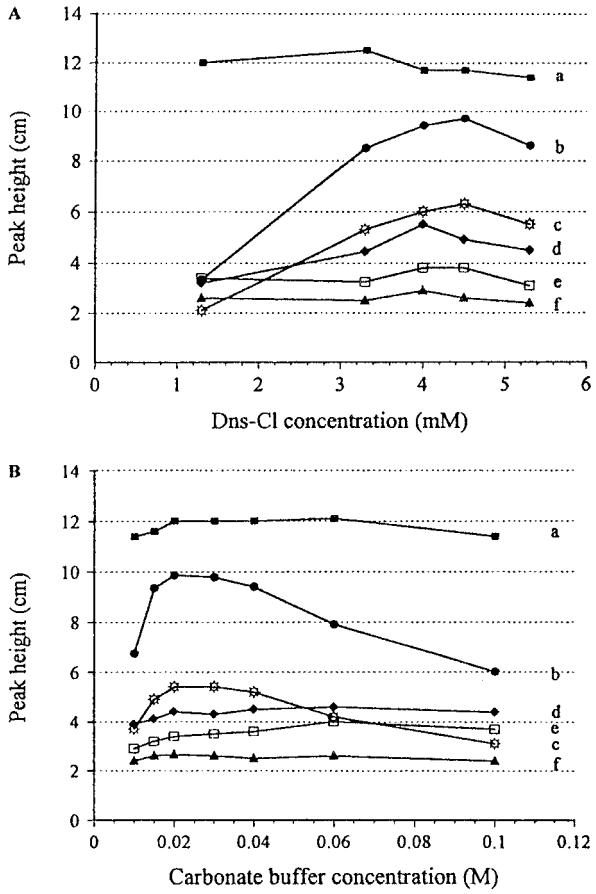

Figure 2. Effects of Dns-Cl (A) and carbonate buffer (B) concentrations on the fluorescence intensity of the Dns derivatives. a) 2-CB; b) Phen; c) Fen; d) E; e) NE; f) 2-PEA.

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This led us to use pH 9.0 at which consistent results with less interference were obtained.

In addition, the effects of temperature and reaction time were examined. Derivatization yield was evaluated at room temperature (RT), 45°C, and 60°C; 45°C proved to be the best temperature where the dansylation yield was higher than that obtained at RT and 60°C for the all compounds. Dansylation increased with time over the intervals examined at 10, 20, 30, 60, 120, 180, 240, and 360 min. Short reaction times were accompanied by the problem of high fluorescence intensity of excess Dns-Cl, which interfered with the separation of NE, E, and 2-PEA under the chromatographic conditions used. This high fluorescence intensity of unreacted Dns-Cl gradually decreased with longer reaction times, and for practical purposes, a 30-min incubation time was chosen.

The excess Dns-Cl was removed by the addition of methylamine, which is highly reactive with Dns-Cl. The reason for adding 3 *M* HCl following methylamine is to neutralize or slightly acidify the strong alkaline pH solution caused by methylamine. This procedure was necessary for the separation of NE from the reagent peak, which was impossible under the high pH conditions.

The Dns derivatives of the compounds studied were shown to be stable for at least 24 h when left at RT.

Finally, the optimized dansylation conditions were fixed as follow: 4.7 m*M* Dns-Cl/0.02 *M* carbonate buffer with pH 9.0, at 45°C and for 30 min reaction time.

Linear calibration curves were obtained under the optimized dansylation conditions for the compounds studied with high correlation coefficients ($r > 0.997$), within the following ranges: for NE and 2-PEA, from 0.25 to 10 μM; for 2-CB from 0.02 to 4 μM; and for E, Phen, and Fen, from 0.05 to 10 μM. The detection limits for the Dns derivatives on the column were 15 fmol for 2-CB, 139 fmol for NE and 2-PEA, and 35, 46, and 49 fmol for E, Phen, and Fen, respectively, at the signal to noise ratio (S/N) of 3.

Selection of an Internal Standard

At the beginning of our work, two internal standards, cyclohexylamine and fluoxetine, were tested. However, in plasma samples, an interfering peak appeared at the same retention time as cyclohexylamine (26 min), which caused us to omit it. On the other hand, FLX gave satisfactory results as an internal standard.

Human Spiked Plasma

Figure 3 shows a chromatogram of Dns derivatives of the compounds studied in human spiked plasma where the separation was obtained within 45 min.

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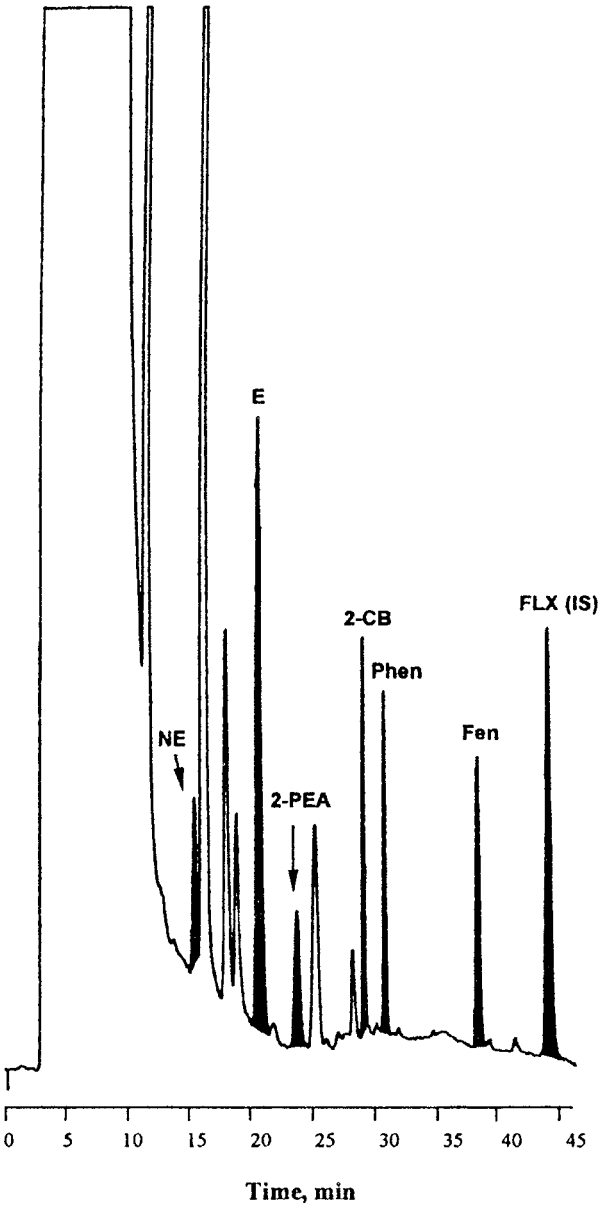

Figure 3. A chromatogram of Dns derivatives for a human plasma sample spiked with 0.25 μM concentrations of NE, E, 2-PEA, Phen, and Fen, and 0.1 μM 2-CB.

Table 1. Linear Ranges, Detection Limits, Recovery, and Intra- and Interassay Validation of Dns Derivatives in Human Spiked Plasma

Compound	Studied Range, μM (r)	Detection Limit, fmol/(μM)	Recovery (%)		Precision[a]	
			0.025 μM	0.5 μM	Intraday	Interday
NE	0.025-2 (0.999)	221 (0.015)	94	91	9.3	10.7 (0.25 μM)
E	0.005-2 (0.997)	36 (0.002)	92	91	5.9	8.8 (0.25 μM)
2-PEA	0.025-2 (0.998)	255 (0.017)	100	94	8.3	10.1 (0.25 μM)
2-CB	0.002-0.8 (0.999)	16 (0.001)	100[b]	96[b]	2.7	4.2 (0.10 μM)
Phen	0.005-2 (1.000)	51 (0.003)	105	103	2.0	2.8 (0.25 μM)
Fen	0.005-2 (1.000)	54 (0.004)	104	99	2.4	2.5 (0.25 μM)

[a]Relative standard deviation %; $n = 4$.
[b]For 2-CB, the recovery was examined at the concentrations 0.01 and 0.2 μM.

Calibration curves were prepared in human plasma spiked with known concentrations of the compounds. The compounds showed good linearity in the ranges studied: for NE and 2-PEA, from 0.025 to 2.0 μM; for 2-CB, from 0.002 to 0.8 μM; and for E, Phen, and Fen, from 0.005 to 2.0 μM. The detection limits at S/N of 3 ranged from 16 to 255 fmol on the column as shown in Table 1.

The recovery of the compounds following the alkaline extraction was examined at two concentrations, 0.025 and 0.5 μM for NE, E, 2-PEA, Phen, and Fen, and 0.01 and 0.2 μM for 2-CB. The recovery was found to be more than 90% and was comparable at the two concentrations studied. The precision of the method was also examined. The intraday precision ($n = 3$) ranged from 2.0 to 9.3% and the interday precision ($n = 4$) ranged from 2.5 to 10.7% as shown in Table 1.

Determination of Phen and Fen in Rat Plasma

The present method was applied for the determination of Phen and Fen in rat plasma after a single intraperitoneal (i.p.) administration of 5 mg/kg of each drug, individually and in combination. Blood samples (200 μL) were drawn into EDTA tubes. The intervals for blood collection were 0, 10, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 300, 360, 480, and 600 min.

An unknown peak appeared in the rat plasma after administration of Fen, which eluted before the peak of Fen at 30 min retention time. We assumed this peak to be an active metabolite of Fen named norfenfluramine (Norfen). However, authentic Norfen is not available in our laboratory; we are now trying to prepare it by an in vitro metabolic method. The results of the metabolic study of Fen will be published elsewhere.

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For the simultaneous administration of both Phen and Fen to rats, overlapping between the peaks of Phen and the unknown compound derived from Fen was expected. Consequently, this led us to modify the chromatographic separation conditions. The mobile phases used in the above method were kept the same, but the gradient time program was modified to that shown in Figure 4, which also presents the separation of the Dns derivatives of the unknown peak, Phen, Fen,

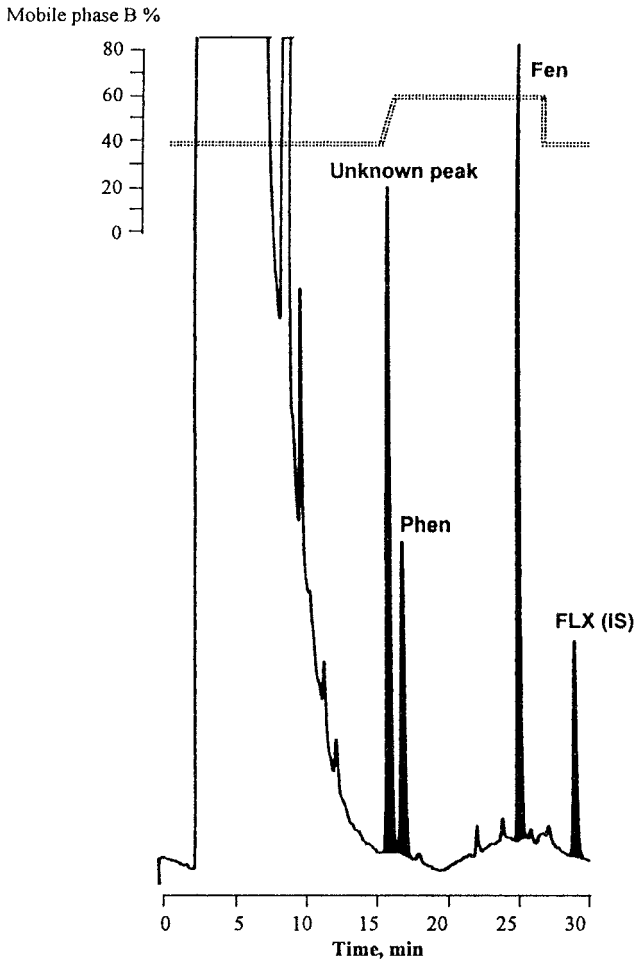

Figure 4. A chromatogram of a rat plasma sample, 30 min after simultaneous administration of a single i.p. dose of 5 mg/kg of Phen and Fen. The Phen peak represents 0.28 μM and the Fen peak represents 0.93 μM.

and FLX for rat plasma samples after administration of a single 5 mg/kg dose of Phen and Fen in combination.

Using the modified system, calibration curves for Phen and Fen were prepared and shown to be linear in the range from 0.005 to 4 μM with correlation coefficients of 0.999 for both drugs and detection limits (S/N = 3) of 54 and 48 fmol on column for Phen and Fen, respectively. The intraday precisions were 2.3 and 4.8% and the interday precisions were 6.3 and 7.8% for Phen and Fen, respectively, for the concentration 1 μM ($n = 4$). Figure 5 presents the plasma levels of both drugs in rat plasma following the simultaneous administration of single i.p. dose of 5 mg/kg of Phen and Fen ($n = 3$).

In conclusion, the present paper describes a simple and sensitive HPLC method for the determination of Phen and Fen in human spiked plasma to the level of 0.003 μM (0.5 ng/mL) and 0.004 μM (0.8 ng/mL) for Phen and Fen, respectively, using small volumes of samples, which makes it more sensitive than methods previously reported. In addition, the method is suitable for the

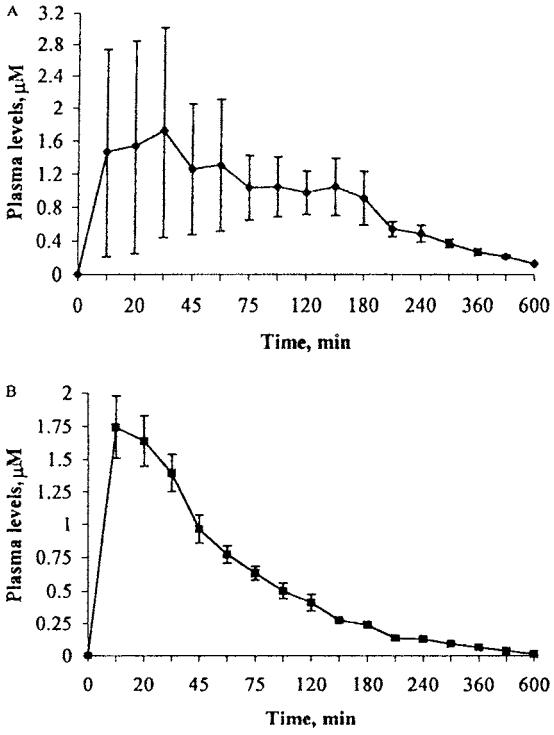

Figure 5. Time course of plasma levels of (A) Phen and (B) Fen after simultaneous administration of single i.p. doses of 5 mg/kg of Phen and Fen to rats ($n = 3$).

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determination of certain other compounds in plasma, namely, NE, E, 2-PEA, 2-CB, and FLX.

Furthermore, the method was shown to be rapid and practical, with the dansylation reaction completed within 30 min and the simultaneous separation of the Dns derivatives accomplished within 45 min. Finally, the method was successfully applied to determine Phen and Fen simultaneously in rat plasma samples dosed i.p. with 5 mg/kg of each.

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